Effect of pyrophosphate ions and alkaline pH on the kinetics of propionaldehyde oxidation by sheep liver cytosolic aldehyde dehydrogenase

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Pyrophosphate ions activate the steady-state rate of oxidation of propionaldehyde by sheep liver cytosolic aldehyde dehydrogenase at alkaline pH values. The steps in the mechanism governing the release of NADH from terminal enzyme·NADH complexes have been shown to be rate-limiting at pH 7.6 [MacGibbon, Buckley & Blackwell (1977) *Biochem. J.* **165**, 455–462]. These steps are shown to be also rate-limiting at more alkaline pH values, and it is through an acceleration of these steps that pyrophosphate ions exert their activation effect.

INTRODUCTION

Previous studies into the effect of pH on the kinetic behaviour of aldehyde dehydrogenases (Blair & Bodley, 1969; MacGibbon *et al.*, 1977b; Takahashi *et al.*, 1981) report an increase in enzymic activity at alkaline pH values. For the mitochondrial forms of this enzyme, the major part of this alkaline-pH activation effect can be attributed to the dissociation of enzyme tetramers into dimeric species, accompanied by an increase in the numbers of functioning active sites (Weiner & Takahashi, 1985). Detailed kinetic studies conducted with sheep liver cytosolic aldehyde dehydrogenase at pH 7.0 and pH 7.6 (MacGibbon *et al.*, 1977a,b,c; Hart & Dickinson, 1982; Dickinson, 1985) have consistently led to the conclusion that only one active site per tetramer is reactive. However, unlike its mitochondrial counterpart, no evidence for dissociation to an activated dimeric form at alkaline pH values has been produced (Dickinson & Allanson, 1985).

The purpose of this study was therefore to investigate further the kinetics of propionaldehyde oxidation at alkaline pH values catalysed by cytosolic sheep liver aldehyde dehydrogenase. The results show that the activation observed in pyrophosphate buffers is a specific effect which increases the rate-determining isomerization of binary enzyme·NADH complexes.

EXPERIMENTAL

Materials

NADH (grade III) and NAD⁺ (grade III) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); propionaldehyde solutions were prepared as described by MacGibbon *et al.*, (1977c).

Methods

Sheep liver cytosolic aldehyde dehydrogenase was prepared essentially by the method of Motion (1986). The enzyme active-site concentration was determined by the method of Bennett *et al.* (1982). Buffers of constant ionic strength (*I* = 0.1 M) were prepared as described by Dawson *et al.* (1969), by using buffers designed by Miller & Golder (1950), as follows: phosphate pH 6.0–7.5, 0.5 M-NaH₂PO₄/4.0 M-Na₂HPO₄/5 M-NaCl; barbitone (sodium 5,5-diethylbarbiturate) pH 8.0–9.0, 2.0 M-sodium barbitone/5 M-NaCl; and for experiments involving pyrophosphate, buffers were maintained at *I* = 0.1 by addition of

NaCl and contained 0.050 M-sodium pyrophosphate. Steady-state enzyme assays were performed as described by Bennett *et al.* (1982) in a Hewlett-Packard 8452 diode array spectrophotometer. Pre-steady-state kinetic experiments monitoring nucleotide fluorescence were performed on a Durrum-Gibson D110 instrument essentially as described by MacGibbon *et al.* (1977a,c) and data were analysed by the methods described by Blackwell *et al.* (1987).

RESULTS AND DISCUSSION

Fig. 1 shows the effect of alkaline pH values on the oxidation of propionaldehyde by the enzyme. Between pH 7 and 9 in a combination of phosphate and barbitone buffers, the rate of propionaldehyde oxidation decreased monotonically with increasing pH, but a marked increase in the rate of propionaldehyde oxidation was observed over this pH range in pyrophosphate buffers. Similar kinetic data to those observed with the phosphate/barbitone buffer systems were obtained with a combination of 100 mM-Hepes (pH 7–8), 100 mM-Tris (pH 7–9), 100 mM-glycine (pH 9.0) and 100 mM-carbonate (pH 9.0). Thus the activation of the enzymic activity observed in pyrophosphate buffers appears to be a specific pyrophosphate-buffer effect.

Steady-state and pre-steady-state kinetic studies with this enzyme (MacGibbon *et al.*, 1977a,b,c; Hart & Dickinson, 1982; Dickinson, 1985) have shown that at pH 7.0 and pH 7.6 a compulsory-order mechanism is followed, as shown below:

where *E·NADH represents a conformationally re-arranged form of the enzyme.

In this scheme the biphasic release of NADH from the terminal enzyme·NADH binary complexes (step 5) is rate-limiting at the neutral pH value of these investigations. The magnitude of the apparent first-order rate constant (λ_s) for the slow phase of NADH release determined in the NADH-displacement experiments sets an upper limit on the rate of the steady-state turnover of the enzyme mechanism (MacGibbon *et al.*, 1977a; Dickinson, 1985).

Fig. 2 shows the variation of the apparent first-order rate constants (λ_s and λ_f) for the slow and fast phases of the decay in

Fig. 1. Effect of pH on the steady-state activity of sheep liver cytosolic aldehyde dehydrogenase

Enzyme ($2.1 \mu\text{M}$), NAD^+ (1 mM) and constant-ionic-strength buffers ($I = 0.1 \text{ M}$) (see under 'Methods') were mixed with $100 \mu\text{M}$ -propionaldehyde, and the steady-state production of NADH was measured by absorbance in a Hewlett-Packard 8452 diode array spectrophotometer (all concentrations represent final concentrations in the assay). Under the above conditions, k_{cat} was determined to be 0.16 s^{-1} at pH 7.0 in the sodium phosphate buffer system. Key: $\circ$, non-pyrophosphate buffers (pH 7.0, sodium phosphate; pH 8.0, pH 9.0, barbitone); $\bullet$, pyrophosphate buffers.

fluorescence which results during displacement of NADH from the enzyme-NADH complexes as a function of increasing pH (the constants λ_s and λ_t are complex functions of the rate constants for step 5 above). The profiles obtained for both decay constants by using the non-pyrophosphate and pyrophosphate buffer systems mirror the apparent k_{cat} profiles of Fig. 1. In the absence of pyrophosphate ions, both λ_s and λ_t decreased with increasing pH in parallel with the decrease in the steady-state rates. In pyrophosphate buffers, however, λ_s increased with pH, and λ_t remained approximately constant with increasing pH. The reversibility of the NADH-release steps (MacGibbon *et al.*, 1977a; Dickinson, 1985) requires that k_{cat} values should always be less than the corresponding λ_s value, and a comparison of the data in Figs. 1 and 2 shows this to be true in all cases. Table 1 gives the relative changes in both apparent k_{cat} values and λ_s values, for both non-pyrophosphate and pyrophosphate buffer systems, as a function of pH. The similarity of the percentage changes in both k_{cat} and λ_s values provides good evidence that the enzyme mechanism is still limited by the release of NADH from the enzyme at alkaline pH values. For non-pyrophosphate buffer systems, increasing the pH leads to a decrease in λ_s and a corresponding decrease in the steady-state turnover of the enzyme. For pyrophosphate buffers, however, the opposite trend is observed, with increasing pH leading to an increase in λ_s and to a corresponding increase in the steady-state turnover.

Fig. 2. Effect of pH on the displacement of NADH from sheep liver cytosolic aldehyde dehydrogenase

Enzyme ($9.0 \mu\text{M}$) and NADH ($40 \mu\text{M}$) in 3.3 mM -phosphate buffer, pH 7.3, were rapidly mixed with NAD^+ (2.5 mM) in the appropriate buffer (see under 'Methods'). All concentrations represent final concentrations in the assay. Key: $\circ$, non-pyrophosphate buffers (pH 7.0, sodium phosphate; pH 8.0, pH 9.0, barbitone); $\bullet$, pyrophosphate buffers. λ_s and λ_t refer to the apparent first-order rate constants for the biphasic displacement of NADH from the enzyme (MacGibbon *et al.*, 1977c). Points represent the mean values ($\pm \text{s.d.}$) of the rate constants obtained from 7–12 repeat runs using the same initial solutions as described above.

Table 1. Comparison of the percentage changes in apparent k_{cat} values and apparent first-order rate constant for the slow step (λ_s) of NADH displacement from the E-NADH complex over the pH range 7–9

Values (means $\pm \text{s.d.}$; $n = 7$ –12) are obtained from the data shown in Figs. 1 and 2. The non-pyrophosphate buffers were sodium phosphate (pH 7) and barbitone (pH 8 and 9).

pH	Non-pyrophosphate buffers		Pyrophosphate buffers	
	k_{cat} (%)	λ_s (%)	k_{cat} (%)	λ_s (%)
7	100	100 ± 8	100	100 ± 5
8	81	82 ± 5	118	119 ± 9
9	56	38 ± 5	263	250 ± 15

Conclusion

The evidence presented shows that the rate of release of NADH from sheep liver cytosolic aldehyde dehydrogenase limits the rate of the oxidation of propionaldehyde by the enzyme at all pH levels between 7 and 9. For all buffers used, except pyro-

phosphate, increasing pH resulted in a decrease in the turnover rate of the enzyme as λ_s decreased. However, the enzyme appeared to behave in an anomalous way in pyrophosphate buffers, in that λ_s now increased as the pH became more alkaline. The exact nature of the pyrophosphate effect on the enzyme remains to be established, but, since λ_s largely reflects the rate of isomerization of binary enzyme-NADH complexes (MacGibbon *et al.*, 1977a), the binding of pyrophosphate ions presumably facilitates this process. This is not an unreasonable suggestion, since Mg^{2+} ions (Bennett *et al.*, 1982) decrease λ_s , and high concentrations of propionaldehyde have been proposed to increase the rate of isomerization (Dickinson, 1985). It could be, therefore, that the isomerization represents a control step for modulating the activity of aldehyde dehydrogenase *in vivo*. Unfortunately it was not possible to measure propionaldehyde oxidation rates accurately above pH 9.0, owing to an alkali-induced non-enzymic reaction between NAD^+ and propionaldehyde (Duncan & Tipton, 1971).

Studies with aldehyde dehydrogenases have involved the use of a wide variety of pH values and a number of buffer types. Some workers have used non-pyrophosphate buffers with pH ranges between 6.0 and 9.0 for the determination of enzymic activity (Kitson, 1981; Harada *et al.*, 1982; Hart & Dickinson, 1982; Agarwal *et al.*, 1985), or a mixture of non-pyrophosphate and pyrophosphate buffers (Takahashi *et al.*, 1981; Alderman *et al.*, 1982; Pietruszko *et al.*, 1985), or pyrophosphate buffers (Koivula & Koivusalo, 1982; Harada *et al.*, 1985), and some groups, although stating the pH used, make no mention of buffer type (Hoe *et al.*, 1985; Woenckhaus *et al.*, 1987). In view of the effects reported in the present paper, caution should be applied when interpreting the results of pH studies on aldehyde dehydrogenases, especially when combining data from pH experiments using pyrophosphate with those for other buffer types.

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